1979

The Biodegradation of Crude Oil by Bacteria Isolated from Brine Pits

Larry Williams
Eastern Illinois University

PAPER CERTIFICATE #2

TO: Graduate Degree Candidates who have written formal theses.

SUBJECT: Permission to reproduce theses.

The University Library is receiving a number of requests from other institutions asking permission to reproduce dissertations for inclusion in their library holdings. Although no copyright laws are involved, we feel that professional courtesy demands that permission be obtained from the author before we allow theses to be copied.

Please sign one of the following statements:

Booth Library of Eastern Illinois University has my permission to lend my thesis to a reputable college or university for the purpose of copying it for inclusion in that institution's library or research holdings.

April 27, 1979
Date

Author

I respectfully request Booth Library of Eastern Illinois University not allow my thesis be reproduced because_____

_____

_____

_____
Date

_____
Author

THE BIODEGRADATION OF CRUDE OIL BY BACTERIA ISOLATED FROM BRINE PITS

(TITLE)

BY

LARRY WILLIAMS

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1979
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
THIS PART OF THE GRADUATE DEGREE CITED ABOVE

27 Apr, 1979
DATE

ADVISER

27 April, 1979
DATE

DEPARTMENT HEAD

The Undersigned, Appointed by the Chairman of the

Department of Zoology,

Have Examined A Thesis Entitled

THE BIODEGRADATION OF CRUDE OIL BY
BACTERIA ISOLATED FROM BRINE PITS

Presented by

Larry D. Williams

A Candidate for the Degree of Master of Science

And Hereby Certify That, In Their Opinion, It Is Acceptable

(Signatures & Date)

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. William Weiler, whose ideas, guidance, and patience made this thesis possible.

Further thanks are expressed to the other members of the author's graduate committee for their help and guidance: Dr. K. D. Baumgardner, Dr. Max Chapman, and Dr. William James.

Sincere thanks are extended to the author's wife, Joann, for her patience and support throughout this graduate study.

ABSTRACT

The Biodegradation Of Crude Oil By
Bacteria Isolated From Brine Pits

Williams, Larry D. M.S., Eastern Illinois University
Major Professor: Dr. William Weiler

The biodegradation of crude oil by bacteria was studied. Hydrocarbon-utilizing bacteria were isolated from sixteen brine pits from an oil producing region of Clark County, Illinois. The bacteria were capable of growth from the vapors arising from crude oil as a sole carbon source. Some strains of bacteria were capable of emulsifying crude oil, apparently through the production of an emulsifying agent(s) that was found to be heat stable and cell bound but which can be partially stripped from the cell by autoclaving. The bacteria appear to belong to the genus Arthrobacter. The chemical environment of the pits was examined and phosphorous levels were suggested as the possible limiting factor in the number of hydrocarbon-utilizing bacteria present.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

FIGURE

TABLE

INTRODUCTION

One of the major ways in which crude oil is removed from the environment is through biodegradation by microorganisms. Microorganisms which degrade crude oil and its derivitives have been studied since the turn of the century. However, due to the increased amount of oil spilled into the environment, these microorganisms have been much more extensively studied in recent years.

In this present study, crude oil-utilizing bacteria were isolated from brine pits located in an oil producing region of Clark County, Illinois. The literature has indicated that certain dissolved inorganic nutrients affect the numbers of hydrocarbon-utilizing bacteria. One of the purposes of this study was to examine the chemical environment of the brine pits in order to determine if and what inorganic nutrients limit the growth of hydrocarbon-utilizing bacteria.

The literature has also indicated that microorganisms degrade crude oil by two basic processes, through utilization of the hydrocarbons of oil for energy and by the production of surface active emulsifying agents which further help break down the oil. In the present study, the isolated bacteria were examined for their

ability to utilize and emulsify crude oil.

There appears to be some conflict in the literature whether the agent(s) responsible for emulsification of crude oil is truly extracellular, that is produced by the bacteria and released into the surrounding medium or whether it is cell bound. A final purpose of this study was to provide data on this aspect of emulsification.

REVIEW OF THE LITERATURE

The Diversity of Hydrocarbon-Utilizing Microorganisms

Hydrocarbon-utilizing microorganisms are ubiquitous in the soil, in the oceans, in fresh and stagnant water and brines, and elsewhere. They are no longer regarded as rare or special types but are widespread in the microbial world (Foster, 1962; McKenna and Kallio, 1964; Sharpley, 1966).

A diversity of organisms exist which can oxidize crude oils and other petroleum hydrocarbons. According to Beerstecker (1954) more than one hundred species of bacteria, yeast, and fungi are able to oxidize hydrocarbons. A partial list of these microorganisms is presented in Table 1. He also states that there is little doubt that the number of species that can utilize hydrocarbons is many times this number and that the ability to assimilate hydrocarbons is a general phenomenon in nature.

A survey of organisms from culture collections made by McKenna and Kallio (1964) revealed that between six and twenty percent of each group of bacteria, yeasts, and molds that were tested were able to grow on n-alkanes. Jones and Edington (1968) made and ecological survey

TABLE 1. Partial Listing of Microorganisms Able to Assimilate Petroleum Hydrocarbons.

Bacteria

Achromobacter	Mulkins-Phillips and Stewart, 1974a; Bryom *et al.*, 1970.
Arthrobacter	Jensen, 1975; Reisfeld *et al.*, 1972; Bryom *et al.*, 1970.
Bacillus	Buckley *et al.*, 1976.
Brevibacterium	Atlas and Bartha, 1972c.
Corynebacterium	Zajic *et al.*, 1977a.
Flavobacterium	Jobson *et al.*, 1972; Atlas and Bartha, 1972c.
Micrococcus	Jobson *et al.*, 1972.
Mycobacterium	Beam and Perry, 1974; Holdom and Turner, 1969.
Nocardia	Mulkins-Phillips and Stewart, 1974a; Beam and Perry, 1974.
Pseudomonas	Jensen, 1975; Jobson *et al.*, 1972.
Vibrio	Mulkins-Phillips and Stewart, 1974a; Beam and Perry, 1974.

Yeasts (from A. J. Markovetz *et al.*, 1964)

Candida lipolytica	*Rhodotorula glutinis*
C. pulcherrima	*R. gracillis*
C. reukaufii	*R. mucilaginosa*
Debaryomyces kloeckeri	*Trichosporon capitatum*

TABLE 1. (cont'd)

Fungi (from A. J. Markovetz et al., 1968)

Cunninghamella sp
C. blakesleeana (+)
C. blakesleeana (-)
Mucor sp
Syncephalastrum sp
Allescheria boydii
A. boydii (M. apiospermum)
A. fumigatus
A. terreus
Botrytis sp
Chaetomium sp
Cephalosporium sp
Cladosporium sp
Fusarium sp
F. solani
Spicaria sp
S. violacea
Geotrichum sp
Gliocladium sp
Helminthosporium sativum
H. tercicum
Oospora sp
Penicillium sp
P. chrysogenum
P. notatum
Phialophora compactum
P. pedrosoi
P. verrucosa
Phoma sp
Pullularia pullulans
Scopulariopsis sp
Sepedonium sp
S. elegans
Trichoderma sp

and coworkers (1943) found that the range of temperatures at which hydrocarbon-utilizing bacteria grow is wide, varying from a little above 0C to 60C. Schwarz and his colleagues (1974) isolated microorganisms capable of utilizing hydrocarbons from Atlantic sediment samples collected at a depth of 4,940 m, corresponding to an *in situ* pressure of 500 atmospheres.

In summary, hydrocarbon-utilizing bacteria are found throughout the world and are not specialized, rare forms. Under normal conditions, it would appear that, although hydrocarbon-utilizing bacteria are common, they are not the dominant type of heterotrophic bacteria. However, in oil-contaminated areas, it has been found that the numbers of hydrocarbon-utilizing microorganisms have increased to the point where they often become the most prevalent type of microorganism (Mulkin-Phillips and Stewart, 1974a).

The Distribution of Hydrocarbon-Utilizing Bacteria

ZoBell (1969) observed that oil-oxidizing bacteria are most abundant in coastal waters and mud where chronic pollution occurs, but such bacteria are extremely scarce in the open sea. Mulkins-Phillips and Stewart (1974a) studied the distribution of hydrocarbon-utilizing bacteria in coastal waters and sediments from the Northwestern Atlantic and found that, depending upon the area's previous history of oil spillage, the percentage of hydrocarbon-utilizing

microorganisms in the total heterotrophic population ranged from less than ten to one hundred percent. However, in the bulk of the samples, they constituted less than ten percent.

Similarly, Atlas and Bartha (1973a) showed a positive correlation between the abundance of oil-degrading microorganisms and patterns of low level oil pollution in Raritan Bay. They suggest that, since a gradient of oil-degrading microorganisms is clearly observed where oil pollution could not be visibly discerned, the presence of oil-degraders seems to be an extremely sensitive indicator of oil pollution.

Davis (1967) uses the fact that soils with a high oil content contain a bacterial population with a high proportion of hydrocarbon-decomposers as a means to explore for underground oil and gas deposits. Perry and Scheld (1968) similarly found that soils from the vicinity of active wells and oil dumps contained significantly greater numbers of hydrocarbon-utilizing microorganisms than samples of garden soil.

Horowitz and Atlas (1977) found that there were more hydrocarbon-utilizing microorganisms in areas of an Arctic freshwater ecosystem that had been heavily contaminated with gasoline than in less heavily contaminated areas. Finally, Sexstone and Atlas (1977) found larger-sized oil-degrading populations in crude oil contaminated Artic soils versus uncontaminated ones.

Factors Affecting Hydrocarbon Utilization

Some of the important environmental factors affecting the distribution of hydrocarbon-utilizing microorganisms include surface area of the oil, oxygen, temperature, salinity, pH, and inorganic nutrient levels (ZoBell, 1969; Abou-Zeid, 1975). Recent studies indicate that temperature and nitrogen and phosphorous levels most often may be the limiting factors in oil biodegradation (Atlas and Bartha, 1972a, 1972b; Ward and Brock,1976).

In a detailed study of a fresh-water lake, Ward and Brock (1976) correlated hexadecane and mineral oil degradation by hydrocarbon-utilizing bacteria with seasonal changes in temperature and dissolved nitrogen and phosphorous levels. They found that during the summer, when temperatures were optimal, nutrient deficiencies limited biodegradation, and higher rates could be obtained by adding nitrogen and phosphorous.

While studying seawater samples treated with petroleum and incubated at controlled temperatures between 5C and 25C, Atlas and Bartha (1972a) found that low water temperature not only resulted in slower degradation, but also an increased lag period before biodegradation began. Under conditions of moderate temperature and aeration, Atlas and Bartha (1972b) observed that low concentrations of phosphate and nitrate in natural sea water were the principle limiting factors of petroleum biodegradation.

The application of urea-phosphate fertilizer has been found to accelerate the biodegradation of crude oil applied to soil and markedly increase the number of bacteria (Cook and Westlake, 1973). Jobson and colleagues (1974) also found that the application of nitrogen and phosphorous (as urea phosphate) resulted not only in a rapid increase in the numbers of bacteria but also accelerated the rate of disappearance of the n-saturate fraction of crude oil applied to the soil.

Nitrogen and phosphorous-containing oleophilic fertilizers have been used to stimulate biodegradation of marine oil slicks (Atlas and Bartha, 1973a). An advantage of such fertilizers is that they selectively supply nutrients to hydrocarbon-utilizing microorganisms and, in contrast to nitrate and phosphate salts, do not trigger algal blooms. Olivieri and coworkers (1976) report similar results using a slow release fertilizer with a paraffin base containing $MgNH_4PO_4$ as the active agent to stimulate oil biodegradation in the marine environment. Twenty-one days after the application of fertilizer, they found 63 percent of the Sarir crude oil had disappeared, compared to 40 percent in the control area without fertilizer.

There appears to be some dispute as to whether nitrogen or phosphorous is the more important limiting factor in the environment (Atlas and Bartha, 1972b; Mulkins-Phillips and Stewart, 1974b). However, Atlas and Bartha

(1972b) report that nitrate or phosphate added individually caused little improvement in oil biodegradation, but when added in combination, they increased petroleum biodegradation to 70 percent.

Iron, too, may play a role in increased biodegradation of crude oil (Dibble and Bartha, 1976). The addition of chelated iron has a stimulatory effect upon the biodegradation of crude oil in relatively iron-poor samples of seawater. Researchers conclude that spills of South Louisiana crude and similar oils can be cleaned up rapidly and efficiently by adding such iron, provided water temperatures are favorable.

Atlas and Bartha (1972a) have proposed a theory to account for decreased biodegradation with decreased temperature. Their results suggest that there is a volatile toxic fraction in Sweden crude oil which is probably present in other crude oils as well. At lower temperatures, the evaporation of this toxic fraction is slowed, thus inhibiting microbial degradation.

Several researchers have found that oil in polluted freshwater and marine sediments can act as a partitioner to concentrate toxic substances such as DDT and other chlorinated hydrocarbon pesticides, polychlorinated biphenols, mercury compounds and other heavy metals. This action results in a highly toxic environment inhibitory to microorganisms attacking the oil (Hartung and Klinger,1970;

Walker and Colwell, 1974a,1976; Sayler and Colwell, 1976).

Other toxic agents include the pollutants hydrogen-sulfide, cyanides from industrial wastes, certain heavy metals, free chlorine (as in heavily chlorinated sewage), and phenols and cresols, all of which may inhibit the growth of hydrocarbon-oxidizing microorganisms (ZoBell,1969). For example, Walker and Colwell (1976) demonstrated that when mercury was added to an oil-containing medium, none of the isolated strains grew, even at a concentration as low as 1ppm.

On the other hand, unwanted biodegradation of various petroleum products can be prevented by the use of toxic inhibitors. Abou-Zeid (1975) lists a number of such inhibitors and methods to prevent biodegradation of petroleum products such as aviation fuel and fuel tank liners.

The mechanics of inhibition are probably variable. Most relate directly to microbial growth, yet Atlas and Bartha (1973c) report the inhibition of crude oil degradation by certain fatty acids produced by two hydrocarbon-utilizing bacteria, members of the genera Brevibacterium and Flavobacterium. They believe that this inhibition represents an end product metabolic control where the crude oil and fatty acids work synergistically.

The Physiology of Petroleum Degradation

Crude oil is essentially a complex mixture of paraffinic, napthenic, aromatic, and asphaltenic hydrocarbons and of nitrogen, sulfur, and oxygen derivitives of these hydrocarbons. There have been many studies to determine which fractions are utilized and what metabolic pathways allow hydrocarbon-utilizing microorganisms to assimilate crude oil. Walker and Colwell (1974b) list a number of methods commonly used to study microbial degradation of petroleum. They state that techniques such as oxygen consumption and biochemical oxygen demand provide a measure of the rate of petroleum degradation. Dispersion and dry weight data are used to indicate the degree of emulsification and the amount of petroleum degraded. Gas-liquid chromatography (GLC) and CO_2 evolution provide information on the rate and extent of petroleum degradation. GLC combined with column chromatography gives information about the class of compounds degraded.

Using these and other methods, it has been found that the paraffinic fraction is possibly the most important component in biodegradation, as it is the fraction that is first metabolized by microorganisms (Miget et al., 1969). Straight and branched alkanes of various chain lengths are all found in the paraffinic fraction of crude oil.

ZoBell (1969) states that, in general, alkanes are

attacked by more microbial species, more rapidly, and support more growth than either aromatic or napthenic compounds. It also has been found that n-alkanes are more susceptible to microbial oxidation than branched ones (Van Der Linden and Thijsse, 1963; ZoBell,1969; Soli and Bens, 1972; Horowitz et al., 1975). ZoBell (1969) also observes that, although certain microorganisms selectively attack unsaturated hydrocarbons, many more exhibit preference for saturated compounds.

Westlake and colleagues (1974), while studying four different crude oils, found that, although the determining factors of biodegradation of crude oil lay primarily in the composition of the n-saturate fraction, other factors such as nitrogen, sulfur, and oxygen content and asphaltene and aromatic fractions also affected biodegradation. Similarly, Stone and coworkers (1942) observed that napthenic hydrocarbons were utilized less readily than paraffinic hydrocarbons but more readily than aromatic hydrocarbons. They emphasized however, that the aromatic fractions proved to be a very acceptable carbon and energy source. Later works have substantiated this fact (Gibson, 1968; Jobson et al., 1972; Westlake et al., 1974).

The ability to utilize petroleum as the sole carbon source connotes a unique and exotic mode of metabolism for hydrocarbon-utilizing microorganisms. This, however has been found to be untrue. Foster (1962) reports that the

only thing unique to the oxidation of hydrocarbons is the breaching of the hydrocarbon with oxygen. Once oxygenated, the molecule enters conventional oxidative pathways.

Many studies have been undertaken to determine what pathways are utilized, some with conflicting results. However, it seems well established that the primary enzymatic attack on alkanes by hydrocarbon-utilizing bacteria occurs at a terminal methyl group (Van Der Linden and Thijsse, 1963; Foster, 1962; Kallio et al., 1963). It is also widely accepted that the alkane is transformed into a fatty acid of corresponding chain length via formation of the primary alcohol and aldehyde, and that the fatty acid is broken down by beta-oxidation, yielding two-carbon fragments, acetyl-CoA (Van Der Linden and Thijsse, 1963; Foster, 1962; Senez and Azoulay, 1963).

Two different mechanisms for the initial attack upon the paraffin molecule are possible. The first involves direct participation of atmospheric oxygen in which the alkane undergoes hydroperoxidation to form alkyl hydroperoxides. The alkyl hydroperoxides subsequently change to n-alcohols, aldehydes, and fatty acids (Figure 1), which then undergo beta-oxidation (Foster, 1962). Long chain alkanes (C12-C18) are apparently oxidized via this pathway (Senez and Azoulay, 1963; Kallio et al., 1963).

A second major pathway for the initial attack upon

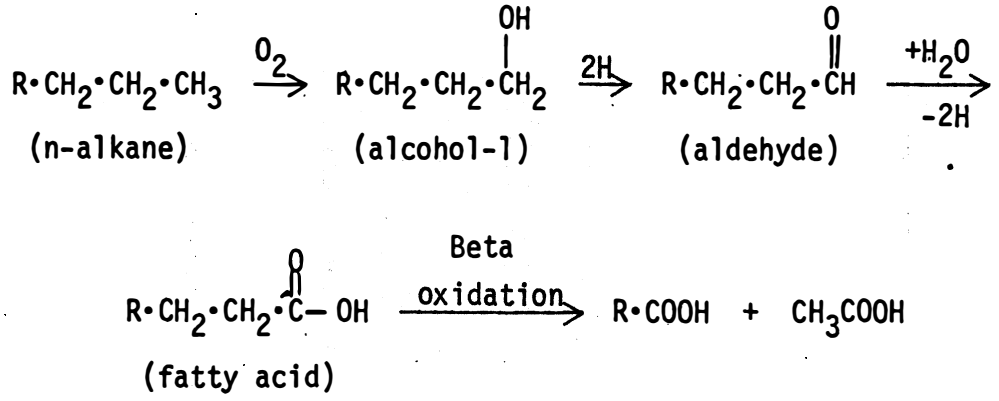

Figure 1. Proposed Scheme For n-alkane oxidation
(Initial attack via atmospheric oxygen)

(Foster, 1962)

alkanes involves anaerobic dehydrogenation of the alkane molecule. The alkane molecule is dehydrogenated to the corresponding 1-alkene (olefin), followed by hydration across the double bond to form the alcohol. Subsequent dehydrogenation occurs to form aldehydes and fatty acids respectively, which then undergo beta-oxidation (Senez and Azoulay, 1963; Kallio et al., 1963). Senez and Azoulay (1963) note that intermediate length alkanes (C6-C10) are metabolized in this matter (Figure 2).

Kallio and coworkers (1963) found that short chain alkanes (C3-C5) produce methyl ketones probably via rearrangement of 1-alkyl hydroperoxides. The methyl ketones are then utilized by the bacteria.

A minor pathway that some hydrocarbon-utilizing bacteria use is that of omega oxidation. In this process, the terminal methyl groups of an alkane or the carbon most remote from the carboxy group in a fatty acid are oxidized to carboxylic acid groups to form diacids, which undergo beta-oxidation (Foster, 1962; Sharpley, 1966).

The pathway in which hydrocarbon-utilizing micro-organisms metabolize aromatic compounds essentially involves the production of a sequence of enzymes which convert the aromatics into an ortho or para-dihydroxy-benzene derivative. This sequence is followed by a series of steps whereby the ring structure is broken and the

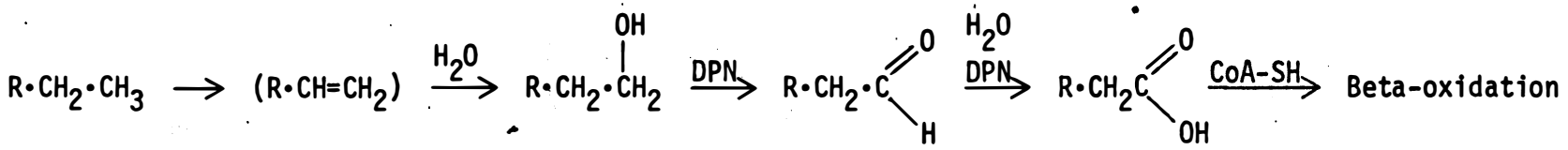

Figure 2. Proposed Scheme For n-alkane oxidation
(initial attack via anaerobic dehydrogenation)

(Senez and Azoulay, 1963)

resulting aliphatic diacids are broken down by beta-oxidation or may enter the Krebs Cycle (Evans, 1963; Gibson, 1968).

Another biochemical system important in the degradation of crude oil is cooxidation. This phenomenon employs essentially two components, the first, a hydrocarbon that at most is used only partially for growth, and the second, a growth substrate, which may or may not be a hydrocarbon. (Raymond et al., 1971). Jobson and coworkers (1972) report increased utilization of aromatics in the presence of whole crude oil which contains more readily utilized saturates. This is important in the natural environment in that it affects the removal of substances which would not be normally utilized to any significant extent (Floodgate, 1972).

The Emulsification of Petroleum

One of the major ways in which crude oil is removed from the marine environment by microorganisms is through emulsification (Zajic and Supplisson, 1972). Two mechanisms of emulsification by microorganisms have been observed (Zajic and Supplisson, 1972).

One of these processes occurs through an increase in the specific gravity and the polarity of the crude oil, caused by oxidation of lower weight hydrocarbons and the entrapment and growth of microbes within the oil emulsion (Jobson et al., 1972; Zajic and Supplisson, 1972).

Such entrapped microbes tend to stabilize small oil droplets by decreasing the surface tension at the oil-water interface.

The other process is through the production of biochemical intermediates or enzymes which act as surface active emulsifying agents (Zajic and Supplisson, 1972). Zajic and his colleagues (1977b) report that to date, few reports on microbially produced emulsifiers have appeared. Some emulsifying agents appear to be truly extracellular while others appear to be cell bound.

While studying an isolated strain of Mycobacterium rhodochorous, Holdom and Turner (1969) found that the emulsifying agent probably was associated with the cell lipid fraction. They also observed that a freeze-dried cell extract was capable of emulsifying decane. Similarly, Zajic and Supplisson (1972) found that freeze-dried mixed bacterial cultures were capable of emulsification of fuel oil.

On the other hand, evidence for extracellular dispersing agents has been observed by several researchers (Reisfield et al., 1972; Zajic et al., 1972, 1977a, 1977b).

Various researchers have described the emulsifying agent as being polysaccharide in nature (Zajic et al., 1974), lipidic (Holdom and Turner, 1969), or a combination of protein, lipid, and carbohydrate (Zajic et al., 1977a, 1977b). Zajic and coworkers (1977a, 1977b) also have

reported that two surface active compounds may be present. Holdom and Turner (1969) have found an agent capable of causing emulsification after being treated in steam at 121C for 30 minutes. Similarly, Gholson and Guire (1973) report that the emulsifying agents isolated were completely stable after boiling in water for 30 minutes. On the other hand, Zajic and his coworkers (1977b) found a significant loss in emulsification when the polymer was treated at a temperature higher than 60C for two hours. Clearly, these different researchers were working with different emulsifying agents.

Theoretically, it would be beneficial to the ecosystem if the emulsifying agents could be isolated and commercially produced. Such agents would be nontoxic and biodegradable. Indeed, Zajic and coworkers (1977b) report that the isolated emulsifying agents were easily biodegraded by two strains of bacteria isolated from the soil.

METHODS AND MATERIALS

General

Glassware

All glassware was washed in commercial detergent (Alconox), followed by thorough rinsing in successive changes of cold water, deionized water, and distilled water.

Sterilization

All glassware and media were sterilized by autoclaving in a Castle autoclave at 121C (15 psi) for twenty minutes. Crude oil was sterilized by heating in a double boiler and refluxing the vapors under a water-cooled cold finger condenser for 2-3 hours on each of three consecutive days.

Media

All media (Appendix A) were prepared in distilled water and sterilized by autoclaving.

Centrifugation

The Sorvall RC2-B automatic refrigerated centrifuge equipped with a 4.25r head was used for all centrifugation procedures.

Incubation

Broth incubation was on a New Brunswick Model G76 Gyrotory Water Bath at 140 RPM and 30C.

Dry heat incubation was at 30C in a Precision Scientific Model 6 Incubator.

Chemical Analysis

All dissolved inorganic nutrients were analyzed with a Hach DR-EL/2 Engineers Lab for water analysis. Reagents used were standard pre-measured and pre-packaged reagents (powder pillows) produced by the Hach Company for use in conjunction with the Hach kit.

Microscopy

All microscopic observations were performed with a Nikon monocular microscope with a 97X oil immersion lens and a 10X eyepiece.

Collection Sites

Sixteen brine pits were sampled. The pits are located in Parker township of Clark County, Illinois, east and west of Illinois Route 49 between Westfield and Oilfield, Illinois.

The pits were classified into three general categories: active, moderately active, and inactive. These categories were based upon the apparent age of the pits, whether crude oil and brine were being pumped into

the pits, the amount of emulsified crude oil sludge in the bottom of the pits, the amount of crude oil floating on the surface of the pits, and the amount of vegetation and the number of animals living in or around the pits.

Active pits are apparently new pits. Brine and crude oil are frequently pumped into them. There is little emulsified crude oil sludge on the bottom and a relatively large amount of crude oil is found on the surface of the water. In addition, there is little or no vegetation or animal life in or around these pits.

Inactive pits are apparently old pits. Little, if any, brine and crude oil are pumped into them. There is substantial emulsified crude oil sludge on the bottom of the pits and little crude oil on the surface. Vegetation (particularly grasses) and animal life are found in and around these pits in relative abundance.

Moderately active pits appear to be somewhere in between active and inactive pits in all the above categories.

Collection of Water Samples

Two different water samples were collected at each brine pit. One sample was collected aseptically in a 500 ml Erlenmeyer flask for bacteriological analysis. The other water sample was collected in a clean 500 ml Erlenmeyer flask for chemical analysis. Both water samples were packed in ice until examination.

Dissolved Oxygen

The dissolved oxygen concentration in the pits was examined at the collecting site at the time the water samples were collected. A YSI Model 54 Oxygen Meter was used to measure the dissolved oxygen.

Microbiological Analyses of Water Samples

Microbiological analyses of the water samples were begun within an hour after collection. Two consecutive decimal (1-10) dilutions of the original sample were made using sterile distilled water as diluent. One-tenth ml of the original water sample and one-tenth ml of each of these two dilutions were pipetted and surface spread onto Bushnell-Haas agar plates (Appendix A). The plates were inverted and a filter pad was placed in the Petri dish top. The pad was then saturated with non-sterile crude oil. The vapors rising from the crude oil provided the sole carbon source for the bacteria (Atlas and Bartha, 1972a).

The plates were incubated for up to three weeks at 30C. Colonies were counted at seven and fourteen days by direct or statistical plate counts. Isolated colonies were restreaked onto Bushnell-Haas plates until pure cultures were obtained.

Pure colonies were inoculated onto Bushnell-Haas agar slants and inverted. A small amount of crude oil was

placed in sponge-rubber foam in the plastic tube closure. Pure cultures were also inoculated onto Plate Count Agar slants (PCA) (Appendix A). These slant cultures were allowed to grow at 30C for seven to fourteen days and were then refrigerated and stored until further use.

Fifty ml of the original water sample was pipetted into each of three 250 ml Delong flasks. One ml of sterile crude oil was pipetted into each flask. The flasks were placed in an incubator for three weeks at 30C. The flasks were checked at seven, fourteen, and twenty-one days for emulsification of the crude oil. Emulsification was determined on a relative scale of 0 to +4.

Chemical Analyses

The water samples were examined for dissolved inorganic nutrients within two hours after collection with a Hach DR-EL/2 Engineer's Lab. The following dissolved inorganic nutrient levels were examined: nitrate nitrite, ammonia, sulfide, and sulfate. The organic phosphate levels were obtained by determining the total phosphate and inorganic phosphate levels and subtracting.

NaCl Analysis

The NaCl concentration in the brine pits was determined with Ames Quantab 1177 Chloride Titrator Strips. These strips have a range of 0.4% NaCl to 12% NaCl. A small sample of the brine was placed in a 50 ml beaker

and a strip was placed in the water sample for fifteen minutes until the strip was saturated. (Saturation was shown by a color change in the strip.) The NaCl concentration was then read directly from the strip and recorded.

Characterization of the Bacteria

Isolated bacteria were taken from storage and restreaked onto PCA plates. To demonstrate purity, two additional PCA streaks were made with each strain.

Colony shape, size, and color on the agar plates were observed and recorded.

Gram stains were made of 6-12 hour cultures, 18-24 hour cultures, and 96 hour cultures on Bushnell-Haas and PCA slants. Microscopic observation of the Gram stains was conducted to determine purity, morphology, and the Gram reaction of the isolate.

The following physiological tests were performed on each of the cultures to help to determine their identity. Citrate utilization was checked using Simmon's Citrate agar. Lactose, sucrose, and glucose utilization were checked in peptone-based Bromthymol Blue fermentation broths. Indole production was examined using tryptone broth. Nutrient gelatin liquification was examined. Acetylmethyl carbinol production and the Methyl Red test were performed in Methyl Red-Voges Proskauer broth. Catalase production was observed on fresh PCA slants.

Sulfide production and the motility of the bacteria were determined in SIM medium. Nitrate reduction was observed in Nitrate Broth. Starch hydrolysis was observed on starch agar.

Crude Oil Emulsification

The bacteria were tested for their ability to emulsify crude oil in the following manner. A loopful of the bacterial isolate was placed into a 250 DeLong flask containing 50 ml of Bushnell-Haas broth (Appendix A). One ml of sterile crude oil was pipetted into the flask. (One ml of sterile crude oil was also placed into an uninoculated control flask containing 50 ml of Bushnell-Haas broth.) The flasks were placed in the shaker bath at 30C and allowed to incubate for one week. The degree of emulsification was determined on a relative scale of 0 to +4.

Characterization of Emulsifying Principle

To determine if the agent responsible for emulsification was extracellular, the following experiment was devised. A loopful of an isolate of a known emulsifying bacterial colony was placed in 50 ml of TGYE broth (Appendix A) and incubated in the shaker bath at 30C for four days. One ml of this culture was then pipetted into each of three 250 ml DeLong flasks containing 50 ml of TGYE broth and one ml of sterile crude oil was placed into

one of the flasks. The flasks were placed in a shaker bath at 30C for four days. Emulsification was noted in the flask containing TGYE, oil, and bacteria.

At the end of four days, the two flasks without crude oil were centrifuged at 9500 rpm (10,000G) for 20 minutes. This effectively separated the bacterial cells from the growth medium. The centrifuged cells from the first flask were resuspended in 50 ml TGYE broth in a 250 ml DeLong flask and one ml of sterile crude oil was added. The supernatant broth was decanted into a sterile 250 ml DeLong flask and one ml of sterile crude oil was added. The centrifuged cells from the second flask were resuspended in 50 ml of Bushnell-Haas broth in a 250 DeLong and one ml of sterile crude oil was added. The flasks were placed on the shaker bath at 30C and observed for emulsification.

In order to determine whether it is necessary for the bacteria to be alive in order to emulsify crude oil and if the emulsifying agent is heat labile, the following two experiments were devised.

In the first experiment, a loopful of the isolate was inoculated into 50 ml of TGYE in a 250 ml DeLong flask followed by incubation in the shaker bath at 30C for four days. The bacteria were pasteurized by placing the flask in a 60-75C water bath for 30 minutes. The culture was centrifuged at 9500 rpm (10,000G) for 20

minutes. The supernatant TGYE was poured into a sterile 250 ml DeLong flask and the bacterial pellet was re-suspended in 50 ml of TGYE in a 250 ml DeLong flask. One ml of sterile crude oil was placed into each of the flasks. The flasks were placed on the shaker bath at 30C for seven days. Flasks were scored for emulsification.

The second experiment was a duplicate of the first with the exception that the bacteria were autoclaved at 121C (15 psi) for fifteen minutes instead of being pasteurized.

RESULTS AND DISCUSSION

The Chemical Environment in Brine Pits

The literature has indicated that dissolved inorganic nutrient levels play an important role in the biodegradation of crude oil by microorganisms. In this study, the inorganic nutrient levels of sixteen selected brine pits were examined, the results of which are summarized in Tables 2 and 3. Similarly, the inorganic nutrient levels of 7 selected freshwater ponds and lakes in the area are presented in Table 4 (Orr, 1975).

The data in this study indicate that, in general, there are greater levels of inorganic nutrients in active brine pits than in inactive ones. Low levels in inactive pits may be partially due to the influx of rainwater with subsequent dilution and leaching. Higher levels in active pits may be due to the occasional "fresh" brine water pumped into them. Another possible explanation for this difference may be utilization of nutrients by microorganisms (Ward and Brock, 1976).

The results indicate that there is considerable variation in the inorganic nutrient levels between individual pits, regardless of classification. This variation can be due to the depth and stratum from which the brines

TABLE 2. The Chemical Environment in Active Brine Pits

Pit Number	2	4	5a	11	13	14	15
NaCl (%)	< 0.42	6.0	< 0.42	< 0.42	1.40	1.40	NR
NITRATE (mg/l)	5.0	2.2	13.2	10.1	1.76	17.6	1.32
NITRITE (mg/l)	< 0.01	0.17	0.03	< 0.01	0.20	0.25	0.02
AMMONIA (mg/l)	2.5	1.13	2.6	9.68	13.5	41.9	21.9
SULFIDE (mg/l)	3.6	0.08	0.27	0.09	0.07	0.39	0.06
SULFATE (mg/l)	24.0	25.0	29.0	7.0	550.0	320.0	310.0
PO_4^a (mg/l)	0.78	0.07	0.67	0.21	0.04	0.8	0.04
PO_4^b (mg/l)	0.00	0.00	0.63	0.30	0.07	NR	0.61
PO_4^c (mg/l)	0.73	0.07	1.30	0.51	0.11	0.8	0.65
DISSOLVED OXYGEN (mg/l)	0.33	9.8	0.40	0.40	5.8	1.4	8.8
SPC* 7 Days	7.4×10^4	2.7×10^3	2.2×10^5	2.4×10^4	1.93×10^3	5.9×10^2	2.3×10^3
SPC* 14 Days	7.4×10^4	3.6×10^3	2.5×10^5	3.8×10^4	1.9×10^3	1.0×10^3	3.1×10^3

NR - Not Run

a - Inorganic Phosphate

b - Organic Phosphate

c - Total Phosphate

SPC* - Standard Plate Count of crude oil utilizing bacteria (Bushnell-Haas agar)

TABLE 3. The Chemical Environment in Inactive Brine Pits

Pit Number	1	3	7	8	9	12
NaCl (%)	< 0.42	< 0.42	0.61	<0.42	<0.42	<0.42
NITRATE (mg/l)	0.9	0.44	1.32	1.32	4.40	0.88
NITRITE (mg/l)	< 0.01	< 0.01	0.23	<0.01	0.14	0.23
AMMONIA (mg/l)	1.42	10.7	0.90	1.61	1.21	1.03
SULFIDE (mg/l)	0.07	0.07	0.04	0.06	0.13	0.06
SULFATE (mg/l)	14.0	26.0	550.0	23.0	100.0	21.0
PO_4[a] (mg/l)	0.03	0.04	0.03	0.02	0.12	0.05
PO_4[b] (mg/l)	0.32	0.21	0.37	0.21	0.63	0.06
PO_4[c] (mg/l)	0.35	0.25	0.40	0.21	0.75	0.11
DISSOLVED OXYGEN (mg/l)	4.0	4.4	8.5	3.2	10.0	8.5
SPC* 7 Days	2.1×10^3	NR	3.2×10^3	4.6×10^4	5.3×10^4	3.2×10^3
SPC* 14 Days	2.6×10^3	1.5×10^4	8.8×10^3	7.0×10^4	9.4×10^4	6.2×10^3

NR - Not Run

a - Inorganic Phosphate

b - Organic Phosphate

c - Total Phosphate

SPC*- Standard Plate Count of crude oil-utilizing bacteria (Bushnell-Haas agar)

TABLE 4.

The Chemical Environment of Selected Freshwater Ponds (Orr, 1975)

	Weiler	Giffin	Williams	Charleston Lake	Campford	Martin	Wapora
Nitrate (mg/l)	0.38	0.10	0.50	10.0	0.04	0.00	4.00
Nitrite (mg/l)	0.001	0.003	0.001	0.055	0.001	0.001	0.001
Ammonia (mg/l)	0.28	0.15	0.25	0.08	0.10	0.20	0.62
Sulfate (mg/l)	14.0	16.0	19.0	52.0	27.0	0.00	0.00
PO_4[a] (mg/l)	0.71	0.40	0.46	0.35	0.23	0.20	0.51
Dissolved Oxygen (mg/l)	10.0	10.4	9.1	8.6	12.7	10.1	9.6

a = inorganic phosphate

were removed (Rogers, 1919), the volume of the brine pits, agricultural runoff from surrounding areas, microbial utilization, or the amount of brine pumped into the pits.

It appears that there is a wider range of dissolved oxygen levels in active pits than the inactive ones. This is probably due to variations in the amount of crude oil pumped into active pits. A thick cover of oil on the surface of the brine would tend to decrease the amount of dissolved oxygen in the water. Inactive pits having little if any crude oil pumped into them seem to show less variation in dissolved oxygen levels.

There did not appear to be any correlation between the dissolved oxygen levels and the reduced forms of nitrate and sulfur. The ammonia levels were generally higher in active pits than in inactive ones and lowest in freshwater ponds. There were very low levels of sulfide in all of the pits. Similarly, levels of nitrite were uniformly low in the brine pits as well as in freshwater ponds.

On the other hand, the data indicate a wide range in sulfate levels in the brine pits. Rogers (1919) grouped brine waters of the Sunset-Midway oil field into three classes. The first class is high in sulfate (309-8790 ppm) and comes from near the surface. The second class is poor or lacking in sulfate and closely resembles seawater. The third class includes waters that are alkaline rather than saline, and free of sulfates. The low sulfate content of

these brines is believed to be due to the direct reducing effect (non-microbial) by the hydrocarbons of petroleum. Bastin et al. (1926) agree with these data but disagree with the explanation for the low sulfate levels. In a study of various oil field waters (including some in the same township as the present study), Bastin et al. (1926) found evidence that the low sulfate levels of the brines are due to the action of sulfate-reducing bacteria. The data in this present study appear to agree with those of Rogers (1919) and Bastin et al. (1926) although they do not give evidence for or against either of the two hypotheses proposed to explain the variation in sulfate levels.

The results of this study indicate that the nitrate levels in active pits are generally higher than those in inactive ones. With one exception (Charleston Lake), the nitrate levels of the freshwater ponds are generally close to the nitrate levels in inactive pits. This may be due to the influx of rainwater (dilution) or the utilization of nitrate by microorganisms (denitrification or sedimentation).

The results show that active pits and freshwater ponds generally contain higher levels of inorganic phosphate than inactive brine pits. Again, this difference may be due to dilution by rainwater or the action of microorganisms. However, evidence against the former idea is illustrated by the fact that phosphate levels of active pits and freshwater ponds are similar. Thus it would appear that

utilization by petroleum-degrading microorganisms may be more important in reducing phosphate levels in brine pits than are physical factors. This also suggests that phosphates may be the limiting inorganic nutrient for oil biodegradation in these pits. Mulkins-Phillips and Stewart (1974b) agree that the rate of natural biodegradation of oil pollutants is limited by phosphate concentrations. However, they and other researchers (Atlas and Bartha, 1972b; Ward and Brock, 1976) emphasize that temperature and nitrate levels also play an important role in petroleum biodegradation.

Classification of Organisms

In the present study, various physiological tests were conducted in order to classify the isolated hydrocarbon-utilizing bacteria. Six unique strains were isolated, varying in at least one physiological characteristic. The results are shown in Table 5.

The Gram reaction and morphology of the bacterial cells were examined at various time intervals, the results of which are shown in Table 6. Snapping division, as evidenced by numerous "V" and "L" forms, was noted in all the cultures. In young (6-12 hr) cultures, the cells appear as Gram negative, irregular short rods. In 24 hour cultures, the cells are also Gram negative rods, but longer and thicker than in younger cultures. Both Gram

TABLE 5. Physiological Characteristics of Crude Oil-Utilizing Bacteria

Test	Strain 16	13	19	b5	a1	11	Reisfeld et al., 1972
Aerobic	+	+	+	+	+	+	+
Citrate utilization	-	-	-	+	+	-	+
Acid from glucose	-	-	-	-	-	-	-
Acid from lactose	-	-	-	-	-	-	-
Acid from sucrose	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-	-
Nutrient gelatin hydrolysis	-	-	-	-	slow	-	+
Nitrate reduction	+	-	-	+	-	+	-
Ammonium utilization	+	+	+	+	+	-	NR
Acetymethyl-carbinol production	-	-	-	-	-	-	NR
Sulfide production	-	-	-	-	-	-	NR
Motility	-	-	-	-	-	-	NR

NR = not reported

TABLE 6. Gram Reaction and Morphology of Crude Oil-Utilizing Bacteria. (PCA at 30C)

Strain	6-12 hr	24 hr	48-96 hr
16	G- bacilli	G- bacilli	G± cocci or very short bacilli
13	G- bacilli	G- bacilli	G± cocci or very short bacilli
19	G± bacilli	G± bacilli	G± cocci or very short bacilli
b5	G- bacilli	G- bacilli	G± cocci or very short bacilli
a1	G- bacilli	G- bacilli	G± cocci or very short bacilli
11	G- bacilli	G- bacilli	G± cocci or very short bacilli

negative coccoid cells or very short rods are characteristic in old (48-96 hr) cultures. In strain 19, both Gram positive and Gram negative rods were noted in the young and 24 hour cultures. All of the cultures were readily decolorized during Gram staining.

In addition, the size, shape, and color of the different bacterial colonies are listed in Table 7. The most active crude oil-emulsifying colonies were muccoid and orange or red pigmented.

Initially, Bergey's Manual of Determinative Bacteriology was used in determining the classification of the isolated strains of bacteria. However, there appears to be some confusion in the literature with regard to the classification of hydrocarbon-utilizing bacteria. Jensen (1975) reports that the coryneforms (including the genera Corynebacterium, Brevibacterium, Arthrobacter, Mycobacterium, and Nocardia) are probably the group most often reported in the literature as showing increases in numbers in the presence of hydrocarbons, but a great deal of confusion still exists with regard to the taxonomy of these genera. Jensen states that it seems quite likely that the strains designated as Corynebacterium, and possibly also Mycobacterium, could as well be referred to as either Arthrobacter or Nocardia.

Reisfeld and coworkers (1972), while studying the microbial degradation of crude oil, listed the identifying

TABLE 7. Colony Description of Crude Oil Utilizing Bacteria. (96 hr at 30C on PCA plates)

Strain	
16	Circular, orange-yellow, muccoid, 2-3 mm in diameter, convex, glistening.
13	Circular, yellow, muccoid, 1-1.5 mm in diameter, convex, glistening
19	Circular, pink-red, muccoid, 2-3 mm in diameter, convex, glistening
b5	Circular, creamy-white, 1-2 mm in diameter, low convex
a1	Circular, bright orange, muccoid, 2-3 mm in diameter, convex, glistening
11	Circular, creamy-white, 1-2 mm in diameter, low convex

characteristics of an oil-emulsifying strain of bacteria which they classified as belonging to the genus Arthrobacter. They state that during the exponential growth phase the cells appear as Gram negative irregular short rods often appearing as "V" shaped pairs. They report Gram positive coccoid cells are characteristic of stationary phase cultures. The physiological characteristics of these bacteria are compared with those isolated in the present study in Table 5. The results appear similar with the exception of nutrient gelatin hydrolysis.

Jensen (1975) reports that there were several slimy orange or red pigmented strains of hydrocarbon-decomposing Arthrobacter species which could not be readily identified as belonging to any of the hitherto described Arthrobacter species.

Thus it would appear that the hydrocarbon-utilizing bacteria isolated in the present study belong to the genus Arthrobacter. However, as Jensen (1975) states, the hydrocarbon-decomposing arthrobacters constitute a group in great need of taxonomic treatment.

Utilization and Emulsification of Crude Oil

Various researchers have found bacteria capable of utilizing the vapors arising from crude oil and other petroleum products as the sole source of carbon (Jones and Edington, 1968; Holdom and Turner, 1969; Atlas and Bartha, 1972a). In the present study, six different strains of

bacteria were isolated from brine pits which were able to utilize crude oil vapors as the only source of carbon (Table 8).

Reisfeld and coworkers (1972) report that of eight different types of bacteria isolated that were capable of growing on crude oil, only one type was able to emulsify crude oil. Similarly, of the six different types of bacteria isolated in the present study, four were capable of emulsifying crude oil (Table 8). It is reported by Midget and coworkers (1969) that two distinct processes may occur during the biodegradation of crude oil. The first is utilization and degradation of oil by the microorganisms and the second is the production of surface-active emulsifying agents. Similarly, Zajic and coworkers (1977) report the quantity of emulsifying agent produced appeared to be growth associated. Therefore, it would appear that some microorganisms, while able to metabolize crude oil, are unable to produce emulsifying agents.

The results of this study indicate that some bacteria can emulsify crude oil more extensively than others (Table 8). This may be because the bacteria are able to produce more surface-active agents than other bacteria or that some surface-active agents may be more effective in emulsifying crude oil.

In this study, the isolated bacterial cultures first showed signs of emulsifying the crude oil in 24-48

TABLE 8. Crude Oil Utilization and Emulsification

Strain	Utilization	Emulsification	
a1	yes	yes	4[a]
b5	yes	no	0
13	yes	yes	4
11	yes	no	0
19	yes	yes	4
16	yes	yes	2

a = degree of emulsification (0-4)

hours. Using isolated cultures, Atlas and Bartha (1972a), noted a 2-4 day lag period before biodegradation occurred. They attribute this delay to the volatile components in Sweden crude oil rather than to a delay in enzyme induction. Assuming biodegradation of petroleum resulting in growth must occur before the elaboration of emulsifying agents, it would appear that the crude oil used in this experiment does not contain such toxic volatile components.

In the present study, experiments were conducted to determine if the emulsifying agents were truly extracellular, that is, produced by the bacteria and released into the surrounding medium, or if the emulsifying agents were bound to the cell. Various researchers have observed evidence for extracellular emulsifying agents (Reisfeld et al., 1972; Zajic et al., 1974; Zajic et al., 1977a, 1977b). However, most studies do not present evidence against cell-bound emulsifying agents. Holdom and Turner (1969) report that the isolated emulsifying factor of Mycobacterium rhodochorous is associated with the cell lipid fraction. Further evidence which suggests that the emulsifying agents may be cell-bound is that freeze-dried cells are capable of emulsification (Holdom and Turner, 1969; Zajic and Supplisson, 1972). The results of the present study are summarized in Table 9 and indicate that the emulsifying agents are primarily cell-bound. Although some emulsification was noted in the supernatant fraction, emulsification

TABLE 9. Characterization of Emulsifying Agents of Crude Oil

Strain	Experiment I centrifuged	Experiment II pasteurized-centrifuged	Experiment III autoclaved-centrifuged
19 cells	4[a]	4	2
19 supernatant	1	1	4
a1 cells	4	4	2
a1 supernatant	1	1	4
16 cells	2	1	2
16 supernatant	1	0	3
13 cells	4	4	2
13 supernatant	1	1	4

a= degree of emulsification

Experimental Protocol

I. Cells and supernatant separated by centrifugation, resuspended, crude oil added to both, placed on shaker bath, observed for emulsification.

II. Cultures pasteurized, cells and supernatant separated by centrifugation, resuspended, crude oil added to both, placed on shaker bath, observed for emulsification.

III. Cultures autoclaved, cells and supernatant separated by centrifugation, resuspended, crude oil added to both, placed on shaker bath, observed for emulsification.

took longer and was not as extensive as in the cell fraction. The cell fraction demonstrated more rapid and more extensive emulsification than the supernatant fraction, even after the cells had been killed by pasteurization. On the other hand, the supernatant fraction demonstrated more extensive emulsification than the cell fraction after the bacterial cultures were autoclaved. This would appear to indicate that the emulsifying agent is heat stable and that autoclaving partially strips the emulsifying agent from the bacterial cells. This is supported by the data of Holdom and Turner (1969) who report an emulsifying factor which is heat stable and released from the bacterial cells by autolysis or by physical means such as sonication and or by treating in steam at 121C for 30 minutes. It would also appear that the emulsifying agent may be associated with the outer surface of the bacterial cell since it can be partially stripped from the cell into the medium to cause emulsification.

Need For Further Studies

With an increasing demand for domestic crude oil, secondary recovery techniques of crude oil will become more widespread. This essentially involves the pumping of water into the wells to force up the crude oil. As a consequence, there will be more brine pits throughout the countryside.

These pits present a possible environmental threat to the surrounding land. In conclusion, there should be further and more extensive studies, particularly concerning hydrocarbon-utilizing microorganisms, to minimize these environmental consequences.

CONCLUSIONS

1. Bacteria isolated from brine pits are capable of growth on an inorganic medium with crude oil vapors as the sole carbon source.

2. Some bacteria isolated from the brine pits are capable of degrading and emulsifying crude oil.

3. The bacteria capable of emulsifying crude oil apparently produce an emulsifying agent(s) that is heat stable. The emulsifying agent(s) is cell-bound, although it can be partially stripped from the cell by autoclaving.

4. The crude oil-emulsifying bacteria may function to assist other microorganisms and physical processes in the removal of crude oil in the brine pits.

5. The crude oil-emulsifying bacteria appear to belong to the genus Arthrobacter.

6. The inorganic nutrient levels of active pits are generally higher than the levels of inactive pits and freshwater ponds, although there is considerable variation in these levels between individual pits. Sulfate levels appear to vary most widely.

APPENDIX A

Media:

1. Plate Count Agar (PCA), TGYE Broth

Bacto-Yeast Extract	2.5 g
Bacto-Tryptone	5.0 g
Bacto-Dextrose	1.0 g
Bacto-Agar	±15.0 g
Distilled Water	1000.0 ml

Autoclaved for 20 minutes, 15 psi

2. Bushnell-Haas Broth/Agar

Magnesium Sulfate	0.2 g
Calcium Chloride	0.02 g
Monopotassium Phosphate	1.0 g
Dipotassium Phosphate	1.0 g
Ammonium Nitrate	1.0 g
Ferric Chloride	0.05 g
Bacto-Agar	±20.0 g
Distilled Water	1000.0 ml

Autoclaved for 20 minutes, 15 psi

Literature Cited

Abou-Zeid, A. 1975. Microbial deterioration of petroleum products and their prevention by toxic inhibitors. Zentralbl. Bakteriol. Parasitenkd. 130: 491-501.

Atlas, R..M. and R. Bartha. 1972a. Biodegradation of petroleum in seawater at low temperatures. Can. J. Microbiol. 18: 1851-1855.

Atlas, R. M. and R. Bartha. 1972b. Degradation and mineralization of petroleum in seawater: limitation by nitrogen and phosphorous. Biotechnol., Bioeng. 14: 309-318.

Atlas, R. M. and R. Bartha. 1972c. Degradation and mineralization of petroleum by two bacteria isolated from coastal waters. Biotechnol, Bioeng. 14: 297-308.

Atlas, R. M. and R. Bartha. 1973a. Abundance, distribution, and oil biodegradation potential of microorganisms in Raritan Bay. Environ. Poll. 4: 291-300.

Atlas, R. M. and R. Bartha. 1973b. Stimulated biodegradation of oil slicks using oleophilic fertilizers. Enviro. Sci. Technol. 7: 538-541.

Atlas, R. M. and R. Bartha. 1973c. Inhibition by fatty acids of the biodegradation of petroleum. Antonie van Leeuwenhoek J. Microbiol. Serol. 39: 257-271.

Bastin, E. S., B. Anderson, F. F. Greer, C. A. Merrit, and G. Moulton. 1926. The problem of the natural reduction of sulphates. Bull. Amer. Assoc. Petrol. Geol. 10: 1280-1299.

Beam, H.W. and J. J. Perry. 1974. Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalism. J. Gen. Microbiol. 82: 163-170.

Beerstecker, E. 1954. Petroleum Microbiology. Elsevier Press, Inc., Houston. 375pp.

Bryom, J. A., S. Beastall, and S. Scottland. 1970. Bacterial degradation of crude oil. Marine Pollution Bull. 1: 25-26.

Buckley, E., R. B. Jonas, and F. K. Pfaender. 1976. Characterization of microbial isolates from an estuarine ecosystem: relationship of hydrocarbon utilization to ambient hydrocarbon concentrations. Appl. Environ. Microbiol. 30: 232-237.

Cook, F. D. and D. W. S. Westlake. 1973. Biodegradability of Northern crude oils. Task force on Northern Oil Development report no. 73-20.

Davis, J. B. 1967. Petroleum Microbiology. Elsevier Press Inc. New York. 604 pp.

Dibble, J. T. and R. Bartha. 1976. Effects of iron on the biodegradation of petroleum in seawater. Appl. Environ. Microbiol. 31: 544-550.

Evans, W. C. 1963. The microbial degradation of aromatic compounds. J. Gen. Microbiol. 32: 177-184.

Floodgate, G. D. 1972. Biodegradation of hydrocarbons in the sea. Pages 153-171 in R. Mitchell, ed. Water Pollution Microbiology. John Wiley and Sons, Inc., New York.

Foster, J. W. 1962. Hydrocarbons as substrates for microorganisms. Antonie van Leeuwenhoek J. Microbiol. Serol. 28: 241-274.

Gholson, R. K. and P. Guire. 1973. Enzymatic removal of oil slicks. National Technical Information Service publication AD-757 071. U. S. Dept. of Commerce.

Gibson, D. T. 1968. Microbial degradation of aromatic compounds. Science 161: 1093-1097.

Hartung, R. and G. W. Klinger. 1970. Concentration of DDT by sediminted polluting oils. Environ. Science and Technol. 4: 407-410.

Holdom, R. S. and A. G. Turner. 1969. Growth of Mycobacterium rhodochorus on n-decane: a new growth factor and emulsifying agent. Appl. Bacteriol. 34: 448-456.

Horowitz, A. and R. M. Atlas. 1977. Response of microorganisms to accidental gasoline spillage in an artic fresh water ecosystem. Appl. Environ. Microbiol. 33: 1252-1258.

Horowitz, A., D. Gutnick, and E. Rosenberg. 1975. Sequential growth of bacteria on crude oil. Appl. Microbiol. 30: 10-19.

Jensen, V. 1975. Bacterial flora of soil after application of oily waste. Oikos 26: 152-158.

Jobson, A. M., F. D. Cook, and D. W. S. Westlake. 1972. Microbial utilization of crude oil. Appl. Microbiol. 23: 1082-1089.

Jobson, A., M. McLaughlin, F. D. Cook, and D. W. S. Westlake. 1974. Effects of amendments on the microbial utilization of oil applied to soil. Appl. Microbiol. 27: 166-171.

Jones, J. G. and M. A. Edington. 1968. An ecological survey of hydrocarbon-oxidizing microorganisms. J. Gen. Microbiol. 52: 381-390.

Kallio, R. E., W. R. Finnerty, S. Wawzonek, and P. D. Klimstra. 1963. Mechanisms of microbial oxidation of alkanes. Pages 453-463 in C. Oppenheimer, ed. Marine Microbiology. C. C. Thomas, publisher, Springfield.

Markovetz, A. J., J. Cazin, and J. E. Allen. 1968. Assimilation of alkanes and alkenes by fungi. Appl. Microbiol. 16: 487-489.

Markovetz, A. J. and R. E. Kallio. 1964. Assimilation of alkanes and alkenes by yeasts. J. Bacteriol. 87: 968-969.

Miget, R. J., C. H. Oppenheimer, H. I. Kator, and P. A. LaRock. 1969. Microbial degradation of normal paraffin hydrocarbons in crude oil. Proc. joint conf. on prevention and control of oil spills. API/FWPCA conf., U. S. Dept. Interior. pp. 327-331.

McKenna, E. J. and R. E. Kallio. 1964. Hydrocarbon structure: Its effect on bacterial utilization of alkanes. Pages 1-14 in H. Heukelekian and N. C. Dondero, ed. Principles and Applications in Aquatic Microbiology. John Wiley and Sons, Inc. New York.

Mulkins-Phillips, G. J. and J. E. Stewart. 1974a. Distribution of hydrocarbon-utilizing bacteria in Northwestern Atlantic waters and coastal sediments. Can. J. Microbiol. 20: 955-962.

Mulkins-Phillips, G. J. and J. E. Stewart. 1974b. Effects of environmental parameters on bacterial degradation of Bunker C oil, crude oils, and hydrocarbons. Appl. Microbiol. 28: 915-922.

Olivieri, R., P. Bacchin, A. Robertiello, N. Oddo, L. Degen, and A. Tolono. 1976. Microbial degradation of oil spills enhanced by a slow release fertilizer. Appl. Environ. Microbiol. 31: 629-634.

Orr, G. L. 1975. A statistical approach to the study of the ecosystems of seven ponds in east-central Illinois. M. S. thesis, Eastern Illinois University.

Perry, J. and H. Scheld. 1968. Oxidation of hydrocarbons by microorganisms isolated from soil. Can. J. Microbiol. 14: 403-407.

Raymond, R. L., V. W. Jamison, and J. O. Hudson. 1971. Hydrocarbon cooxidation in microbial systems. Lipids 6: 453-457.

Reisfeld, A., E. Rosenberg, and G. Gutnick. 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl. Microbiol. 24: 363-368.

Rogers, G. S. 1919. The Sunset-Midway oil field, California. Professional paper 117, U. S. Geological Survey.

Sayler, G. S. and R. R. Colwell. 1976. Partitioning of mercury, and polychlorinated biphenyl by oil, water, and suspended sediment. Environ. Sci. and Technol. 10: 1142-1145.

Schwarz, J. R., J. D. Walker, and R. R. Colwell. 1974. Deep sea bacteria: growth and utilization of hydrocarbons at ambient and *in situ* pressure. Appl. Microbiol. 28: 982-986.

Senez, C. S. and E. Azoulay. 1963. Pathways of lower alkane oxidation by *Pseudomonas*. Pages 464-474 in C. Oppenheimer, ed. Marine Microbiology. C. C. Thomas, publisher, Springfield.

Sexstone, A. J. and R. M. Atlas. 1977. Response of microbial populations in artic tundra soils to crude oil. Can. J. Microbiol. 23: 1327-1333.

Sharpley, J. M. 1966. Elementary Petroleum Microbiology. Gulf Publishing Co., Houston. 385 pp.

Soli, G. and E. M. Bens. 1972. Bacteria which attack petroleum hydrocarbons in a saline medium. Biotechnol., Bioeng. 14: 319-330.

Stone, R. W., M. R. Fenske, and A. G. C. White. 1942. Bacteria attacking petroleum and oil fractions. J. Bacteriol. 44: 169-178.

Van Der Linden, A. C. and G. J. E. Thijsse. 1963. Degradation of 2-methylhexane by a Pseudomonas. Pages 475-480 in C. Oppenheimer, ed. Marine Microbiology. C. C. Thomas, publisher, Springfield.

Walker, J. D. and R. R. Colwell. 1974a. Mercury-resistant bacteria and petroleum degradation. Appl. Microbiol. 27: 285-287.

Walker, J. D. and R. R. Colwell. 1974b. Microbiol petroleum degradation: Use of mixed hydrocarbon substrates. Appl. Microbiol. 27: 1053-1060.

Walker, J. D. and R. R. Colwell. 1976. Oil, mercury, and bacterial interaction. Environ. Sci. Technol. 10: 1145-1147.

Walker, J. D., R. R. Colwell, and L. Petrakis. 1975b. Degradation by an alga, Prototheca zopfii. Appl. Microbiol. 30: 79-81.

Walker, J. D., R. R. Colwell, and L. Petrakis. 1976. Biodegradation of petroleum by Chesapeake Bay sediment bacteria. Can. J. Microbiol. 22: 423-428.

Walker, J. D., R. R. Colwell, Z. Vaituzis, and S. A. Meyer. 1975a. A petroleum degrading achlorophylous alga, Prototheca zopfii. Nature 254: 423-424.

Ward, D. M. and T. D. Brock. 1976. Environmental factors affecting the rate of hydrocarbon oxidation in temperate lakes. Appl. Environ. Microbiol. 31: 764-772.

Westlake, D. W. S., A. Jacobson, and R. Phillippe. 1974. Biodegradability and crude oil composition. Can. J. Microbiol. 20: 915-928.

Zajic, J. E., H. Guignard, and D. F. Gerson. 1977a. Emulsifying and surface active agents from Corynebacterium hydrocarboclastus. Biotechnol., Bieong. 19: 1285-1301.

Zajic, J. E., H. Guignard, and D. F. Gerson. 1977b. Properties and biodegradation of a bioemulsifier from Corynebacterium hydrocarboclastus. Biotechnol., Bioeng. 19: 1303-1320.

Zajic, J. E. and B. Supplisson. 1972. Emulsification and degradation of Bunker C fuel oil by microorganisms. Biotechnol., Bioeng. 14: 331-343.

Zajic, J. E., B. Supplisson, and B. Volesky. 1974. Bacterial degradation and emulsification of No. 6 fuel oil. Environ. Sci. and Technol. 8: 664-668.

ZoBell, C. E. 1969. Microbial modification of crude oil in the sea. Proc. joint conf. prevention and control of oil spills API/FWPCA, Dept. of Interior. pp. 317-326.

ZoBell, C. E., C. W. Grant, and H. F. Haas. 1943. Marine microorganisms which oxidize hydrocarbons. Bull. Amer. Assn. Petrol. Geol. 27: 1175-1193.